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PHASE VARIATION IN SALMONELLA[1]

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THE flagella of most Salmonella serotypes occur in two alternative, clonally stable phases. Earlier applications of transductional technique have shown that the phases correspond to two loci, H_1 and H_2 (LEDERBERG and EDWARDS 1953). The present study is concerned with the internal control of the alternation.

1. HISTORICAL BACKGROUND

1.1 Serological analysis of Salmonella dates to the differentiation of antigenic components in motile and nonmotile strains of *S. cholerae-suis* by SMITH and REAGH (1903). They and subsequent authors have classified these components as an "H" thermolabile, flagellar and an "O" thermostable, somatic antigen respectively. Further analysis has proceeded by the now familiar techniques of cross-absorption of antiserums prepared against various strains, and has led to the elaborate tabulation known as the KAUFFMANN-WHITE diagnostic scheme, part of which is abstracted in table 1. The scheme is an important guide in epidemiological tracing of Salmonella infections, and newly discovered types are therefore promptly recorded and deposited with International Salmonella Typing Centers. The cooperative efforts of hundreds of bacteriologists therefore make a wealth of information and natural variants available for genetic study.

1.2 The most striking feature of the scheme is its combinatorial pattern—*prima facie* evidence for a mechanism of genetic recombination which encouraged eventually successful efforts at its demonstration (LEDERBERG 1947, 1948; ZINDER and LEDERBERG 1952). The serotypes entered in the table can be construed as generated by the combination of three basic elements, the somatic antigen complex and the two flagellar phases. As we will not be immediately concerned with the former it will be symbolized only by the conventional groups, rather than detailed formulae. However, such abbreviations denote antigenic patterns whose genetic interest is no less than that of the flagellar factors (cf. UETAKE *et al.* 1955).

1.3 Flagellar phase variation was discovered by ANDREWES (1922) in *S. typhimurium*. Mass cultures of this serotype may agglutinate in either of two H-antisera, anti-i or anti-1.2. A reisolated clone might react only, say, with i. On further cultivation, the i clone will generate an occasional offshoot which reacts only with 1.2. In its turn, the 1.2 phase is liable to throw off an occasional i, an alternation which will be symbolized i:1.2. The variation from one phase to another may occur at any

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time, with a small probability per bacterial division, which ranged from 10^{-5} to 10^{-3} in the strains studied by STOCKER (1949). In rapidly varying strains, phase variation can be detected by random colony tests; in more stable material, selection by the immobilizing action of antiserum against the existing phase is effectively practiced.

1.4 While its stochastic indeterminacy tends to signify phase variation as mutation, it is bounded by two fixed alternatives which are characteristic of the serotype. As shown in table 1, any phase-1 antigen may be associated with any phase-2, and the one cannot be predicted from the other. The table also shows that the flagellar antigens can be divided into two groups, viz., phase-1: a, b, c, d, eh, i, r, etc., and phase-2: 1.2, 1.5, 1.7, enx, etc. One antigenic alternative is chosen from one group, one from the second. This pattern of variation has no parallel in the mutation of other genes, and until recombinational analysis was applicable, genetic interpretations of phase variation could only be speculative (DUBOS 1945; LEDERBERG 1948).

1.5 The genetic relationship of the phases in a wide variety of serotypes has been investigated by transductional techniques (LEDERBERG and EDWARDS 1953). For example, the transduction from *S. typhimurium*, i:1.2, as donor, to *S. abony*, b:enx as recipient gave the new combinations i:enx and b:1.2, but not such types as i:b or

TABLE 1

The KAUFFMANN-WHITE scheme of Salmonella serotypes, condensed from KAUFFMANN (1951)

Salmonella serotype	Somatic antigen	Flagellar phase-1	Antigens phase-2	Salmonella serotype	Somatic antigen	Flagellar phase-1	Antigens phase-2
kisangani	B	a	1.2	manhattan	C2	d	1.5
arechavaleta	B	a	1.7	newport	C2	eh	1.2
bispebjerg	B	a	enx	kottbus	C2	eh	1.5
tinda	B	a	enz_{15}	takoradi	C2	i	1.5
abortus-equi	B	—	enx	bonariensis	C2	i	enx
paratyphi B	B	b	1.2	bovis-morbificans	C2	r	1.5
abony	B	b	enx	hidalgo	C2	r	enz_{15}
wagenia	B	b	enz_{15}	sendai	D	a	1.5
altendorf	B	c	1.7	loma-linda	D	a	enx
stanley	B	d	1.2	onarimon	D	b	1.2
saint-paul	B	eh	1.2	typhi	D	d	- -
chester	B	eh	enx	ndolo	D	d	1.5
essen	B	gm	—	eastbourne	D	eh	1.5
typhimurium	B	i	1.2	enteritidis	D	gm	- -
heidelberg	B	r	1.2	shoreditch	D	r	enz_{15}
san juan	C1	a	1.5	butantan	E1	b	1.5
oslo	C1	a	enx	shangani	E1	d	1.5
edinburg	C1	b	1.5	elisabethville	E1	r	1.7
cholerae-suis	C1	c	1.5	marseille	F	a	1.5
mission	C1	d	1.5	chandans	F	d	enx
lomita	C1	eh	1.5	aberdeen	F	i	1.2
montevideo	C1	gms	- -	venezziana	F	i	enx
virchow	C1	r	1.2	mississippi	G	b	1.5
narashino	C2	a	enx	heves	H	d	1.5
gatuni	C2	b	enx	hvittingfoss	I	b	enx
muenchen	C2	d	1.2				

1.2:enx, which would have been as readily detectible by the selective method. These and many analogous results imply that the phase-1 antigens form one genetic homology group, the phase-2 a second. The factors within each group are mutually interchangeable by transduction, while factors of different groups are not.

1.6 In a word, each group is the set of alleles of an H_1 and H_2 locus, respectively, so that the phenotype b:enx corresponds to the genotype $H_1^b H_2^{enx}$. Furthermore the two loci are independently transduced, i.e., they are not closely linked to one another for the i:1.2 class was also not produced. If the two antigenic phases are thus determined at separate loci, phase variation cannot be construed as the mutation from one specific allele to another. Instead, it is the alternative manifestation of each of the two antigenic specificities already inherent in the genotype. This concept was already provoked by the closed cycle of variation emphasized above.

1.7 To investigate this alternation, experiments were directed primarily at the inheritance of flagellar phase in recombinant clones generated by transduction. Where the phase of a culture was determined, it will be indicated by bold face: thus, b:**enx** signifies a clone displaying the enx antigen that varies to the **b**:enx form, in which the b antigen is now manifest.

1.8 Recombinational analysis in Salmonella (so far) depends on a system of transduction (the transmission of hereditary fragments) mediated in this instance by bacteriophage particles (ZINDER and LEDERBERG 1952). The leading work on this system involved a variety of nutritional, fermentative and resistance markers, and was followed by studies on mutants affecting the formation and action of flagella (STOCKER *et al.* 1953) and on the flagellar antigens (LEDERBERG and EDWARDS 1953). Every marker that was studied and was technically feasible has been subject to transduction at a more or less uniform incidence (cf. ZINDER 1955, for quantitative comparisons of transduction efficiency of different factors). The symbolism A ⟶ x B or B x⟵ A will be used to indicate a transduction with A as donor, B as recipient, i.e., the application of a cell-free lysate of A to intact cells of B.

2. MATERIALS AND METHODS

2.1 Transduction was carried out with lysates of Salmonella phage PLT22 grown on indicated host cultures as previously described, 1.8

2.2 Two modes of selection were used to isolate the minority of transduction clones from the excess background of unaltered recipient cells. Both methods take advantage of a soft nutrient gelatin agar (NGA) through which motile bacteria readily swarm, while nonmotile or immobilized cells grow in place. (A), Mixtures of bacteria and phage were inoculated on NGA plates to which antiserum for both phases of the recipient bacteria was added. These are therefore immobilized, but any bacteria which manifest a different H antigen, viz., one transduced from the donor, swarm from the point of inoculation. For example, outgrowing swarms of **b**:1.2 and i:**enx** from b:enx ⟶ x i:1.2 can be picked twelve to twenty-four hours after NGA plates containing i and 1.2 antiserum are inoculated. (B), Alternatively, a *Fla*$^-$ (flagellaless, nonmotile) recipient was used in NGA without antiserum. Individual Fla$^+$ swarms were then typed for their flagellar antigens. The application of this procedure to the

present problem depends on the demonstrated linkage of *Fla* and H_1 factors (STOCKER *et al.*, 1953; 3.4, 3.7).

2.3 Serotypes were characterized by routine methods (EDWARDS and EWING 1955) of slide agglutination in 1:100 antiserum. Live cell suspensions were emulsified from colonies or used directly from penassay broth cultures. A number of the serotypes have been confirmed by tube agglutination tests in several laboratories. When the alternative phases were not both obvious on routine tests, they were ascertained after selection in NGA with antiserum against the existing phase. Most of the serums were standard reagents, i.e., sterile cresolated preparations of rabbit antiserums that had been absorbed to remove somatic and heterologous antibodies, with a tube agglutination titer of at least 1:10,000. They were used at a dilution of 1:1000 for selection in NGA.

2.4 The principal markers for this study were the naturally occurring alleles of H_1 and H_2 in the following stock cultures (STOCKER *et al.* 1953; LEDERBERG and EDWARDS 1953): *S. typhimurium*, TM2, i:1.2; *S. abony*, CDC-103, b:enx; and *S. heidelberg*, SL-28, *Fla*$^-$ r:1.2. *Gal*$^-$ (galactose-negative) mutants of *S. typhimurium* and of *S. abony* were obtained after UV-irradiation and scored on EMB galactose agar (LEDERBERG 1950). Additional *Fla*$^-$ mutants were obtained in *S. typhimurium* from UV-irradiated suspensions by plating them in NGA at a density of one to two hundred survivors per plate. The plates were inoculated three to six hours at 37°C to allow motile clones to initiate small swarms. They were then held an additional twelve to eighteen hours at 25°C at which temperature the gelatin would set and restrict further swarming. However, the clones could continue to grow to an easily visible density in situ. The *Fla*$^-$ mutants were then readily distinguished as compact spherical colonies, in contrast to the diffuse nebulae of the parental *Fla*$^+$ clones. Eight *Fla*$^-$ mutants were isolated, of which five were sufficiently stable to be tested further.

3. RESULTS AND CONCLUSIONS

3.1 *Serum selection; mixed recipients.* The leading precedent for alternative manifestation of serotypic potentialities is undoubtedly the study of *Paramecium aurelia* by SONNEBORN and his coworkers (BEALE 1954) whereby the cytoplasm was shown to be propagated in one of several inter-transformable states which determine the pattern of genic expression. If a principle of complete cytoplasmic determination were applicable to Salmonella phase variation, the phase should be inherited entirely from the recipient in transduction, in view of the disproportionate mass of the intact cell in comparison with that of the phage-borne fragment. The first experiments were therefore designed to establish whether the donor phase had any influence on the issue of transduction. Phage lysates were prepared from single colony cultures of *S. typhimurium* (i:1.2) whose purity of phase was controlled by slide agglutination tests on twenty or more colonies. All cultures used in these experiments consisted of at least ninety percent of cells in the indicated phase, and were more often entirely homogeneous over the sample of twenty subclones. For the recipient, an equal mixture of **b**:enx and b:**enx** cells of *S. abony* was used so that the only variable would be the phase of the donor. The phage and cells were mixed and planted on NGA agar plates containing b and enx antiserum, and the resulting swarms isolated

and characterized as follows:

		i:enx	b:**1.2**	Total swarms tested
a)	i:1.2 —x b:enx	19	0	19
b)	i:**1.2** —x b:enx	14	14	28

For this 2 × 2 table, $\chi^2 = 15$, and $P < .01$. Similar results were obtained in repetitions of this design, and it was concluded that the donor phase does influence the results of transduction, contrary to the cytoplasmic hypothesis.

3.2 The absence of b:1.2 types in experiment a) above might be explained by the inherent nontransducibility of $H_2^{1,2}$ from phase-1 cells in which it is not expressed, or by the persistence of its inactivity in the recipient. On the latter view, the transduction of $H_2^{1,2}$ would result in a **b**:1.2 recombinant, which would not swarm, however, due to its inhibition by the anti-b serum. That is, in these experiments, a new genotype will only be detected if it inherits the appropriate phase as well as antigenic potentiality. Some more direct substantiation for this assumption will be offered in the following sections.

3.3 *Motility selection: H_1 linked to Fla.* From the influence of the donor phase 3.1 it can be concluded that the control of phase is associated with the transduced fragments, i.e., that it is linked either to H_1 or H_2 or both. The following experiments were designed as further tests of the assumptions of 3.2, by studying the inheritance of phase in transductions of H_1 as an unselected marker linked to a selected *Fla*$^+$ factor.

3.4 The first of these experiments used SL 28, the naturally occurring *Fla*$^-$ mutant of *S. heidelberg*, *Fla*$^-$ r:1.2. Phase variation in this culture cannot be detected by phenotypic difference, as no flagella or flagellar antigens are produced, but can be determined after *Fla*$^+$ has been restored either by (rare) reversions or transduction from another strain. Preliminary trials of *S. abony* —x SL28 were made on fresh single colony cultures, and two of them were saved when their motile progeny were preponderantly **r**:1.2 and r:**1.2** respectively. Each phase was then used as a recipient with lysates from *S. abony*, **b**:enx and b:**enx** respectively, according to the procedure of 2.2B with results shown in table 2.

3.5 Columns 1 and 2 indicate the transductions of *Fla*$^+$ uncoupled with H_1^b and verify the stability of the two phases in the recipient. Columns 3 and 4 show the transductions in which *Fla*$^+$ was coupled with H_1^b . This was immediately discernible

TABLE 2

Transduction of H_1^b *coupled with* Fla$^+$ S. abony
Fla$^+$ *b:enx* —*x* S. heidelberg Fla$^-$ *r:1.2*

Phase of transductional parents		Fla$^+$ transductional clones				
Donor	Recipient	(1) r:1.2	(2) r:**1.2**	(3) **b**:1.2	(4) b:**1.2**	Total tested
b:enx	**r**:1.2	21	0	22	0	43
b:enx	r:**1.2**	0	7	1	42	50
b:**enx**	**r**:1.2	11	0	30	0	41
b:**enx**	r:**1.2**	1	10	1	38	50

for **b**:1.2 types, and could be determined after the alternative phase was scored in b:**1.2** vs. r:**1.2** types. The b:**1.2** type is especially important as it verifies directly that a b phase can be transduced in an inactive form. In the experiments conducted by antiserum selections (3.1, 3.10), such recombinant clones would be suppressed.

3.6 Table 2 also shows that the recipient governs the yield of types, and the donor phase has no effect. This apparent conflict with the previous results can be resolved by recalling that in 3.1 both H_1 and H_2 transductions were enumerated, here only H_1. There is also an unexplained difference in the frequency of linked and unlinked transductions in the different combinations. It is not known whether the discrepancy is systematically related to the phases of the donor and recipient, or is simply a casual peculiarity of a single lysate. No such effect was noted in the comparable experiment of table 4.

3.7 To complete an experimental design similar to 3.4 and table 2 in the more closely studied system *S. abony* —x *S. typhimurium*, a number of *Fla*⁻ mutants of the latter were examined for linkage to H_1 and H_2. Each *Fla*⁻ mutant was tested x— *S. abony* **b**:enx and b:**enx**, and *Fla*⁺ progeny examined for the presence of the b and enx antigens. Three of the eight *Fla*⁻ mutants reverted too readily to be useful in these experiments. Of the other five, one, SW-1157 showed occasional coupling of *Fla*⁺ with H_1^b (table 3). None showed linkage of *Fla* with H_2. The *Fla*⁻ mutants were also tested against one another and with a number of other *Fla*⁻ stocks and found to transduce motility to one another in all combinations, i.e., they carry *Fla*⁻ mutations at distinctive loci (cf. STOCKER *et al.* 1953).

TABLE 3

Linkage relations of Fla⁻ *mutants from* S. typhimurium *i:1.2*

Mutant	*Fla*⁺ selections			
	Fla⁻ x— *Fla*⁺ b:enx tests *Fla-H*₁ linkage		*Fla*⁻ x— *Fla*⁺ b:**enx** tests *Fla-H*₂ linkage	
	b:1.2	i:1.2	i:**enx**	i:**1.2**
1	0	39	0	16
2	0	48	0	28
3	0	12	0	7
4	0	35	0	15
5 (SW-1157)	5	40	0	28

TABLE 4

Transduction of H_1^b *coupled with* Fla⁺ S. abony Fla⁺ *b:enx* —*x* S. typhimurium *SW1157* Fla⁻ *i:1.2*

Phase of transductional parents		*Fla*⁺ transductional clones				
Donor	Recipient	(1) i:1.2	(2) i:**1.2**	(3) b:1.2	(4) b:**1.2**	Total tested
b:enx	**i**:1.2	80	1	14	0	95
b:enx	**i**:**1.2**	3	86	0	27	116
b:**enx**	**i**:1.2	68	4	18	0	90
b:**enx**	**i**:**1.2**	1	90	0	20	111

3.8 In a further search for technically useful linkages, a series of twenty-six auxotrophic markers (DEMEREC, BLOMSTRAND, and DEMEREC 1955) was tested for linkage to the *Fla-H*$_1$ segment. *Fla*$^+$ selections from the various *Aux*$^-$ *Fla*$^+$ —x *Aux*$^+$ *Fla*$^-$ transductions were tested, but each of 1352 isolates was still *Aux*$^+$ (prototrophic), implying that none of these markers is linked to *Fla*.

3.9 The experiment outlined 3.4 was repeated with *S. abony* —x SW-1157, with entirely analogous results, except that the proportion of linked H_1^b-*Fla*$^+$ transductions was uniformly lower (table 4).

3.10 *Serum selection, labelled recipients.* The next experiments were a study of all four combinations of donor and recipient phase under serum selection. In order to improve the comparability of the experiments, the recipient was a mixture of phases, distinguished by a *Gal* marker. That is, from a *Gal*$^-$ mutant of *S. typhimurium*,

TABLE 5

Transductions of S. abony *and* S. typhimurium
Serum-selection; Gal-labelled recipients

i:1.2 —x b:enx; b, enx serum						b:enx — i:1.2; i, 1.2 serum					
Donor	Recipient		Transduction clones			Donor	Recipient		Transduction clones		
	Phase	Gal	Gal	i:enx	b:1.2		Phase	Gal	Gal	b:1.2	i:enx
i:1.2	**b**:enx	+	+	15	0	**b**:enx	**i**:1.2	+	+	10	0
	b:**enx**	−	−	1	0		i:**1.2**	−	−	0	0
i:**1.2**	**b**:enx	+	+	6	8	b:**enx**	**i**:1.2	+	+	4	16
	b:**enx**	−	−	2	11		i:**1.2**	−	−	0	34
i:1.2	**b**:enx	−	−	10	0	**b**:enx	**i**:1.2	−	−	16	1
	b:**enx**	+	+	0	0		i:**1.2**	+	+	0	0
i:**1.2**	**b**:enx	−	−	10	7	b:**enx**	**i**:1.2	−	−	6	19
	b:**enx**	+	+	1	8		i:**1.2**	+	+	0	29

TABLE 6

Summary of all experiments similar to table 5

Donor	Recipient	Relative incidence of transduction clones			
		Table 5		All experiments	
		$\mathbf{d_1}$:r_2	r_1:$\mathbf{d_2}$	$\mathbf{d_1}$:r_2	r_1:$\mathbf{d_2}$
$\mathbf{d_1}$:d_2	$\mathbf{r_1}$:r_2	51	1	127	7
$\mathbf{d_1}$:d_2	r_1:$\mathbf{r_2}$	1	0	3	2
d_1:$\mathbf{d_2}$	$\mathbf{r_1}$:r_2	26	50	108	155
d_1:$\mathbf{d_2}$	r_1:$\mathbf{r_2}$	3	82	3	185

colonies in either i:1.2 or i:**1.2** phase were picked, and the pure-phase culture mixed with the alternative phase from the *Gal*+ strain. The mixed cells were then treated with phage from *S. abony* and inoculated on NGA plates with i and 1.2 serum. The resulting transduction clones (either i:**enx** or b:1.2) were also scored for their Gal character. An analogous experiment was also conducted with the reciprocal transduction. Previous trials had shown no indication of linkage between *Gal* and the *H* markers, so that the Gal character of a new combination told the phase of the parental recipient for each transductional clone. The results are given in detail in table 5. In table 6, the corresponding results from a number of similar experiments are pooled, d_1:d_2 signifying the generalized donors, and r_1:r_2 the recipients. The qualitative features of table 6 may be summarized as follows:

	Donor	Recipient	Manifest transductions	Probable missing classes
a.	**d_1**:d_2	**r_1**:r_2	**d_1**:r_2	**r_1**:d_2
b.	**d_1**:d_2	r_1:**r_2**		**r_1**:d_2 d_1:**r_2**
c.	d_1:**d_2**	**r_1**:r_2	r_1:**d_2** **d_1**:r_2	
d.	d_1:**d_2**	r_1:**r_2**	r_1:**d_2**	d_1:**r_2**

3.11 In this summary, the rare entries are disregarded, and may be considered to arise from a minor admixture of phase in the parents. The r_1:**d_2** types seem also to outnumber the **d_1**:r_2. This discrepancy might reflect a minor difference in the efficiency of transduction of the H_1 and H_2 loci by the lysates used, or in the recovery of the two types under the conditions of selection. As indicated earlier, the missing classes are presumed to occur but are suppressed because they retain the antigenic phase of the recipient parent, for which antiserum is present in the NGA. The excess unaltered parental cells restrict the total growth of the transduction clones at the site of inoculation so that they lack an opportunity to engender phase variants that can be detected. The occurrence of the missing d_1:**r_2** type was verified in previous experiments, 3.5, 3.9; the **r_1**:d_2 is hypothetical so far, owing to the lack of any means of selecting for it (e.g., another marker linked to H_2).

3.12 *Hypothetical expectations.* A number of hypotheses may be considered for their concordance with the experimental results and are listed in table 7. The only hypothesis among those listed that agrees with the data is number 5, that the "state" of the H_2 locus (or one closely linked to it) governs the phase. It is difficult to establish whether the list is exhaustive, but the following restatement may help to expose the implicit assumptions. Since the donor phase does influence the result of transduction of the *H* factors, we assume that some element associated with the factors themselves is involved. If the phage particle carries no other relevant material besides chromosome fragments, the state of the fragment itself is in question. Of the relevant possibilities, the experimental results are clearly in favor of a decisive role of the H_2 locus. This hypothesis is amplified, and the possible coordinate role of cytoplasm and other factors is discussed in 4.1.

3.13 *Consistency of the experiments.* The earlier experiments can be considered as special cases of 3.10. 3.1 can be derived by lumping the two phases of the recipient, and is qualitatively consistent with it: compare the proportions 19: 0: 14: 14 with the corresponding 130: 9: 111: 340. Tables 2 and 3 can be correlated by considering

TABLE 7

Hypotheses of phase variation and their consequences

Hypothesis	Genotype of		Types expected from							
	$r_1:r_2$	$r_1:\mathbf{r_2}$	$\mathbf{d_1}:d_2$ —x $\mathbf{r_1}:r_2$		$\mathbf{d_1}:d_2$ —x $r_1:\mathbf{r_2}$		$d_1:\mathbf{d_2}$ —x $\mathbf{r_1}:r_2$		$\mathbf{d_1}:\mathbf{d_2}$ —x $r_1:\mathbf{r_2}$	
			1	2	1	2	1	2	1	2
1. Cytoplasmic state	$H_1^rH_2^r\gamma^1$	$H_1^rH_2^r\gamma^2$	$\mathbf{d_1}:r_2$	$-\mathbf{r_1}-$	$-\mathbf{r_2}-$	$r_1:\mathbf{d_2}$	$\mathbf{d_1}:r_2$	$-\mathbf{r_1}-$	$-\mathbf{r_2}-$	$r_1:\mathbf{d_2}$
2. Controlling gene	$H_1^rH_2^rG^1$	$H_1^rH_2^rG^2$	$\mathbf{d_1}:r_2$	$-\mathbf{r_1}-$	$-\mathbf{r_2}-$	$r_1:\mathbf{d_2}$	$\mathbf{d_1}:r_2$	$-\mathbf{r_1}-$	$-\mathbf{r_2}-$	$r_1:\mathbf{d_2}$
3. Complementary states of H_1 and H_2	$\boldsymbol{H_1^rH_2^r}$	$H_1^r\boldsymbol{H_2^r}$	$\mathbf{d_1}:r_2$	$-\mathbf{r_1}-$	$\mathbf{d_1}:r_2^*$ $-\mathbf{r_2}-$	$r_1:\mathbf{d_2}^\ddagger$ $-\mathbf{r_1}-$	$\mathbf{d_1}:r_2^\ddagger$ $-\mathbf{r_2}-$	$r_1:\mathbf{d_2}^*$ $-\mathbf{r_1}-$	$-\mathbf{r_2}-$	$r_1:\mathbf{d_2}$
4. State of H_1	$\boldsymbol{H_1^rH_2^r}$	$H_1^rH_2^r$	$\mathbf{d_1}:r_2$	$-\mathbf{r_1}-$	$\mathbf{d_1}:r_2$	$r_1:\mathbf{d_2}$	$-\mathbf{r_2}-$	$-\mathbf{r_1}-$	$-\mathbf{r_2}-$	$r_1:\mathbf{d_2}$
5. State of H_2	$H_1^rH_2^r$	$H_1^i\boldsymbol{H_2^r}$	$\mathbf{d_1}:r_2$	$-\mathbf{r_1}-$	$-\mathbf{r_2}-$	$-\mathbf{r_1}-$	$\mathbf{d_1}:r_2$	$r_1:\mathbf{d_2}$	$-\mathbf{r_2}-$	$r_1:\mathbf{d_2}$
Experimental results Table 6			$\mathbf{d_1}:r_2$ 127	$r_1:\mathbf{d_2}$ 7	$\mathbf{d_1}:r_2$ 3	$r_1:\mathbf{d_2}$ 2	$\mathbf{d_1}:r_2$ 108	$r_1:\mathbf{d_2}$ 155	$\mathbf{d_1}:r_2$ 3	$r_1:\mathbf{d_2}$ 185

The symbols $-\mathbf{r_1}-$ in columns 2 and $-\mathbf{r_2}-$ in columns 1 stand for $\mathbf{r_1}:d_2$ and $d_1:\mathbf{r_2}$ respectively. In experiments like table 6, which are done in the presence of r_1 and r_2 antiserums, these types would not be recovered.

The various hypotheses are defined by the genotypes which each assigns to the two recipient phases. The corresponding donor genotypes may be written, *ex hypothesi*, by substituting the *d* for the *r* allelic symbols. The expected types are inferred by substituting H_1^d in the indicated state for H_1^r in columns 1, and H_2^d for H_2^r in columns 2. The phenotype of each transduction is then determined from the postulated genotype.

‡* Auxiliary assumptions are required to make predictions from this hypothesis when two active* or two inactive‡ loci are combined by transduction. The table shows expectations when the choice of phase from this impasse is random. On other assumptions, the results of the two tests should be similar, unless the hypothesis is reduced to a case of No. 4 or 5 by assuming that one locus outweighs the other.

only the relative incidences of $\mathbf{d_1}:r_2$ in table 6, and ignoring the transductions of H_2 listed in the $r_1:\mathbf{d_2}$ column; then compare 22: 1: 30: 1 and 12:0: 13: 0 with 127: 3: 108: 3, respectively, which constitute excellent agreement on the principal issue of the role of the recipient (*viz.* the H_2 locus) in the activity of transduced H_1 factors.

3.14 *Monophasic types.* Although diphasic behavior is more prevalent, a number of Salmonella types are monophasic, e.g., *S. typhi* d:—; *S. enteritidis* gm: —; *S. abortus-equi* —:enx. Such types are especially valuable for the production of specific serums, and have been sought after for that reason (EDWARDS and BRUNER 1946). Curiously, the natural types carrying g and related antigens are almost always monophasic, despite the fact that diphasic combinations of these factors, e.g., gp:1.2 are readily synthesized by transduction. The ecological selective factors that must be involved in this peculiarity are unknown.

3.15 Apparent monophasic behavior may be complicated by serological cross-reactions between the phases. For example, *S. cholerae-suis*, var. *kunzendorf* is recorded as —:1.5 or (c):**1.5**, as selection with anti-kunzendorf 1.5 serum is occasionally but sporadically successful for the demonstration of the c phase (EDWARDS and BRUNER 1946). However other 1.5 and 1.2 serums are more consistently effective.

The different activity of different serums might be taken as evidence that they have a specific inductive as well as a selective effect on phase variation. However, the c and 1.5 phases of this serotype are shown (LEDERBERG and EDWARDS 1953, unpublished) to have a common antigen, agglutinins for which are present in the anti-kunzendorf 1.5 serum and which also tend to suppress the c as well as the 1.5 phase. This antigen (which might be a somatic rather than a flagellar component) had been disregarded previously because it is unusually labile to formalin, commonly used in setting up H-agglutination tests.

3.16 For other monophasic types, e.g., *S. abortus-equi* (a):**enx**, this contingency probably does not apply, but the hidden phase can be demonstrated either by stringent selection for rare variations (EDWARDS and BRUNER 1939) or by transduction of the H_1^a allele to other recipients (LEDERBERG and EDWARDS 1953). This type therefore has the genotype $H_1^a \boldsymbol{H_2^{enx}}$ but some factor is present at these or other loci which stabilizes the existing phase, and is under current study.

3.17 *S. typhi* represents still a third variety of monophasic behavior which can be represented by the genotype H_1^d H_2-deficient, for no homology with H_2 factors of other strains can be detected by transductions in either direction, although the H_1 factor is readily transduced and substituted for. *S. enteritidis*, H_1^{gm}, and related types, and some varieties of "monophasic *S. paratyphi B*", H_1^b, appear to belong in the same category.

3.18 A fourth category is represented by another strain of "monophasic *S. paratyphi B*", CDC-137, which can be described as $\boldsymbol{H_1^b}$ H_2^- where "$-$" implies a null allele always hypostatic to H_1. In transductions to other strains, this type never transfers an effective H_2 allele. However, it readily incorporates transduced H_2 factors, e.g., $\boldsymbol{H_1^b}$ H_2^- x— $H_1^a \boldsymbol{H_2^{enx}}$ to give a typically diphasic $H_1^b H_2^{enx}$. This type may be the counterpart of *S. abortus-equi* (3.16) since an absolutely stabilized H_2 factor in the hypostatic state could never be expressed, even if transduced elsewhere.

3.19 These types are enumerated to display some of the wealth of material now open for recombinational study in the Salmonella group. They also show how monophasic exceptions can be accomodated to the pattern of phase control which was deduced from typical diphasic forms.

3.20 *Antigenic mutation.* The technique of immunoselection can also be used for the selective isolation of new mutant alleles at a single locus, once the genetic structure of diphasic types has been worked out. For some time, it has been known that some serotypes which carry b occasionally generate an "artificial phase" antigen, z_{33}, when selected in b serum. Without further analysis, z_{33} might be interpreted either as a mutation $H_1^b \rightarrow H_1^{z33}$, or a phase variant in $H_1^b H_2^{z33}$. In the course of the present study, the following changes have been obtained as isolated events during selection in the homologous serums: *S. paratyphi B*, **b**:1.2 $\rightarrow$ $\mathbf{z_{33}}$:1.2, and *S. abony*, **b**: enx $\rightarrow$ $\mathbf{z_{33}}$:enx. The derived types showed typical phase variation between the indicated phases. They were also subjected to transductions to and from **b**:1.2, **b**:enx, and **i**:1.2 in which z_{33} proved to be homologous with b and i respectively. This suggests that z_{33} corresponds to the allele H_1^{z33}, and that the change from H_1^b to H_1^{z33} is a true mutation. The z_{33} shows, however, a strong antigenic relationship to the b antigen, and further studies on the differentiation of the b and z_{33} alleles should be preceded by a more detailed serological analysis of the stocks in question.

3.21 The following reports of antigenic variations should also probably be classified as mutations of the H_1 or H_2 locus as indicated, although they have not yet been analysed by transduction tests. H_1 changes: d → j; (see KAUFFMANN 1951) lv → lw (BRUNER 1954); and a complex series gm → gp, mt → gt, mt → gms, gmq → gm, gmq → gq, gmt → mt, gmt → gt, gmt → gm, and gst → gs (BRUNER 1953). H_2 changes: 1.2 → 1.2.3, 1.2.3 → 1.2 (BRUNER and EDWARDS 1948); 1.5 → 1.10, 1.5 → 1.11 (BRUNER and EDWARDS 1947).

3.22 Although the symbols for these antigens are more complex than those for b and z_{33}, they are not fundamentally different, as the formulae primarily reflect a more detailed analysis. In general, the antigenic mutations have generated forms still related to the primitive form, and mutations of the scope, for example, of b → i have yet to be observed.

4. DISCUSSION

4.1 The preceding account has supported the conclusion that phase variation can be explained by an alternation of "states" of the H_2 locus. When this gene is in the active or epistatic state, its allele is phenotypically expressed, and the H_1 factor is functionally suppressed. Alternatively, when the H_2 gene is in the inactive or hypostatic state, the H_1 factor is expressed. While the H_1 and H_2 factors are thus functionally complementary, the H_1 factor does not vary in its *heritable* state, and its activity depends on the state of the H_2 locus. Therefore, when an inactive H_1 is transduced, from a donor in phase-2 where H_2 was epistatic, to a recipient in phase-1 wherein H_2 is hypostatic, the H_1 factor can be immediately expressed. The experiments have thus indicated that the "state" of the H_2 factor is inherited as such with the transduction of H_2 specificity, while the functional state of H_1 is not. The primacy (or stability of state) of the H_2 locus in transduction does not preclude a more integrated system during vegetative growth in which the cytoplasm or the H_1 locus may still play an important role in the perpetuation of phase. For example, epistasis by H_2 might depend on local saturation by the immediate products of its action, a steady state or feedback condition which would depend in turn on the functioning of the cytoplasm and the competitive inefficiency of a stripped H_1 locus. Unfortunately, we have almost no facts on which to build speculations on the material meaning of "state": one of the first unknowns to be pursued might well be the *external* regulation of phase. Phase variation (more accurately phase stability) evidently does not depend on the actual production of the antigenic end product, since it can be demonstrated in *Fla*$^-$ mutants which are devoid of H antigens (STOCKER *et al.* 1953; 3.4, 3.7).

4.2 The flagellar antigens are known to be proteins (STOCKER 1956) but in Salmonella they have not been studied in regard to structural features such as amino acid composition, or even their basic physicochemical properties, so there is no fundamental basis for distinguishing the phases except by their serological reactivity. However, phase-2 flagella are differentially agglutinated by acridine dyes (BERNSTEIN and LEDERBERG 1955) which speaks for some overall difference in the action of H_1 and H_2. In addition, some strains show differences in motility between the two phases (EDWARDS *et al.* 1954); whether this reflects a quantitative or qualitative difference in the flagellar proteins is a nice question. There is no precise evidence on

the regulation of phase variation by external influences, partly because of technical difficulties (cf. STOCKER 1949). There has been considerable discussion of "induced phases" in Salmonella, but no evidence that antiserum plays other than a selective role in the detection of serotypic changes has been forwarded.

4.3 Throughout this paper we have referred to *serotypes* notwithstanding the use of binomial epithets and frequent allusions elsewhere to *species* of Salmonella. Bacterial taxonomy has been dominated by the principle that any clonally stable variety whose characters can be sharply differentiated constitutes a species, a definition which is at variance with the conventions for higher forms. Transductional analyses have shown that serotypes differ in regard to well defined unit markers, but the extent to which genic differences diffuse among bacterial populations in nature is unknown.

4.4 The characterization of serotypes in this work has depended on the KAUFFMANN-WHITE scheme and on the reagents prepared to its specifications. Its authors have cautioned that the scheme is an abbreviation for diagnostic purposes, and not a complete exposition of the antigenic individuality of the serotypes. For example, the symbol "d" does not always betoken an identical antigenic constitution, and minor differences are often detectable by cross-absorption tests. The extent to which such differences are symbolized (e.g. in the distinction of enx from enz_{15} in contrast to the lumping of distinguishable 1.5 phases) depends on two possibly gratuitous factors, as well as historical accidents: the apparent titers of the residual antiserums after cross-absorption, and the urgency of more detailed differentiation as an element of classification. Neither of these considerations stands out as a necessary correlate of the importance of "minor" differences for genetic analysis. Unfortunately, the minor differences are not systematically catalogued, although they are usually mentioned in original publications of types. The sheer bulk of the present scheme has encouraged a more recent lumping of related serological phases, e.g., 1.2 with 1.2.3, rather than a finer analysis. The immediate practical consequence of these cautions is that the conventional instructions for the preparation of reagents should be followed explicitly rather than be substituted for by seemingly plausible deductions from the diagnostic scheme (KAUFFMANN 1951; EDWARDS and EWING 1955).

4.5 In the original classification of *Fla*$^-$ mutants (STOCKER *et al.* 1953), three were found to be closely linked to H_1 but were not identical. Many other *Fla*$^-$ mutants were examined and found to be nonallelic with one another and not linked to H_1. Further studies have shown that *S. heidelberg* SL28 also carries an H_1-linked *Fla*$^-$ mutation, as do several additional isolates that were not available earlier, including the laboratory mutant of *S. typhimurium* SW-1157, table 3. These *Fla*$^-$ mutants are all distinct from one another. Evidently, one cluster of *Fla*$^-$ mutants occurs in the neighborhood of the H_1 locus. Unfortunately, there is no reliable direct method of demonstrating linkage of one *Fla* factor to another, owing to their identity of phenotype. The non-H_1-linked *Fla*$^-$ mutants might therefore represent another compact cluster, or they might be widely dispersed (at least they are not closely linked to H_2 by a hypothetical analogy). It is impossible to judge whether the linkage of *Fla* factors to H_1 is fortuitous, or is somehow interrelated with their action on a common organelle (cf. DEMEREC *et al.* 1955). The appearance of a clusters of closely linked, nonrecurrent mutants with the same phenotype is reminiscent of the cluster of

Lac$_I$ and of *Gal* mutants in *Escherichia coli* (E. M. LEDERBERG 1952; MORSE *et al.* 1956). Allelism tests on the more limited scale of conventional technique in, say, Drosophila would probably have described each of these clusters as a set of multiple alleles at a single locus.

4.6 For the moment, the *H* factors appear to be simple alleles, but these markers are not so readily screened for rare recombinants, and they may yet prove to be compound in the sense of the histocompatibility factors in mice (GORER, unpubl., cited in ALLEN 1955). The persistent cross-reactivity of the original and variant in antigenic mutations such as b $\rightarrow$ z_{33} or 1.5 $\rightarrow$ 1.10 is the only hint in favor of this speculation, and the H_1 factors so far have retained their integrity in recombinational analysis. Preliminary chemical studies encourage the view that antigenic components of a complex such as enx are manifestations of different aspects of the same flagellar protein (NAKAYA *et al.* 1952).

4.7 In a system of DNA-mediated transduction (transformation) in *Hemophilus influenzae*, LEIDY, HAHN and ALEXANDER (1953) have described the separation of capsular determinants a and b in a fashion to suggest that these are nearly but not precisely allelic. In the pneumococcus, EPHRUSSI-TAYLOR (1951) had likewise demonstrated a series of closely linked "subunits" which control the quantitative aspects of capsular polysaccharide synthesis. Her "autogenic" transformations are readily interpreted as linked transductions of the factor complex, the "allogenic" transformations as transductions of a part only, i.e., as crossing over within the complex.

4.8 The state of a locus can be considered as a parameter of genic variation independent of mutational changes in specificity, which recalls D. LEWIS' (1954) account of fractional changes in self-compatibility genes in Oenothera. These mutant genes had lost only their function in the pollen, but retained their function and specificity in the style. The suppression of phenotypic effects of transposition of heterochromatin (E. B. LEWIS 1950) or of the *Ds* and *Mp* elements (MCCLINTOCK 1956; BRINK and NILAN 1952) may also be viewed as local changes of state, which although they depend on structural modifications nevertheless suggest how gene action may be regulated. These may therefore be considered as conceptual, if not mechanical, models of phase variation.

4.9 The cytoplasm has attracted considerable favor as the place of morphogenetic differentiation, largely because of an argument which has been well rephrased by EPHRUSSI (1953): "Unless development involves a rather unlikely process of orderly and directed gene mutation, the differential must have its seat in the cytoplasm". Most experiences with genic changes have involved sporadic, irreversible changes in specificity that qualify them very poorly for a role in development. However, the concept of local states may provide a more acceptable hypothetical basis for nuclear differentiation. This reconsideration of the nucleus has recently been bolstered by the caryonidal determination of mating type in Tetrahymena (NANNEY and CAUGHEY 1955), morphological diversification of chromosomal regions during the development of various tissues (see BREUER and PAVAN 1955), and especially the direct demonstration by KING and BRIGGS (1955) with nuclear transplantations from cells in later to earlier stages in frog development. The interplay of coordinate nuclear and cytoplasmic determiners has been emphasized by SONNEBORN (1954).

SUMMARY

1. The flagellar antigens of Salmonella types occur in two alternative phases, e.g., i and 1.2 in *S. typhimurium*. Transduction analysis had shown that each phase corresponds to a distinct locus, so that the genotypic formula of *S. typhimurium* would be $H_1^i\ H_2^{1,2}$. Phase variation therefore consists of the alternative manifestation of antigenic potentialities at these loci.

2. Transduction experiments were carried out with *S. typhimurium*, *S. abony* b:enx, and *S. heidelberg*, r: 1.2, with donor and recipient in various phases. The expression of the H_1 loci depended on the phase of the recipient (which retained the original H_2 locus), while the expression of a transduced H_2 locus depended on the phase of the donor cell. It is therefore concluded that antigenic phase is governed by H_2 locus, which occurs in either of two clonally stable (or nearly stable) states. The concept of "local state" presents a parameter of genic variation distinct from ordinary mutation, and may be applicable to other problems in genetics and morphogenesis.

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